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# CORTICAL RESPONSE ANTIGENS RELEASED AT FERTILIZATION FROM SEA URCHIN EGGS AND THEIR RELATION TO ANTIGENS OF THE JELLY COAT [1]

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The roles of specific macromolecules in the cortical response of the sea urchin egg at fertilization are complex and incompletely understood. This lack of understanding is evident when one considers the variety of functionally distinct substances which have been reported to be released during the cortical response and which may be associated with one or more types of cortical granules or cortical cytoplasm. These substances have been variously described as a sperm agglutinin ("cytofertilizin," Motomura, 1950), the precursors of the fertilization acid ("fertilization product," Ishihara, 1964, 1968ab), a membrane toughening factor ("colleterin," Motomura, 1957), a jelly precipitating factor ("antifertilizin" or JePptF (E)," (Runnström, Wicklund and Löw, 1954), a membrane elevating factor ("MbElF (E)", Runnström *et al.*, 1954), a polyspermy preventing factor (Sugiyama, 1951), the hyaline layer material (Nakano, 1956), an activating substance ("A-antigen," Perlmann and Perlmann, 1957) and an enzyme ($\beta$-1, 3-glucanase, Epel, Weaver, Muchmore and Schimke, 1969).

A necessary step toward an understanding of the functional significance of the cortical response must involve some means of making qualitative distinctions among the various macromolecules released from the egg at the time of fertilization. The present work was undertaken with the aim of using specific antibodies as analytical reagents for such a qualitative assay. The work reported here concerns the possible identity of "cytofertilizin" with fertilizin (the sperm agglutinin of egg jelly) as well as the relationship between "cytofertilizin" and the "fertilization product." Parts of this study have been reported in preliminary form elsewhere (Gregg and Metz, 1966; Metz, 1967).

## Material and Methods

### *Spawning*

The sea urchin, *Arbacia punctulata*, was used in this study. Urchins were obtained from the Marine Biological Laboratory Supply Department at Woods

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Hole, Massachusetts, or commercially from Mr. Glendle W. Noble, Florida Marine Biological Supply Company, Panama City, Florida. The animals were induced to spawn using electrical shock. Semen was shed directly into dry syracuse watch glasses and eggs were shed into filtered sea water.

*Removal of egg coats*

Prior to fertilization or activation, eggs were either dejellied with acidified sea water (final pH 5.0) or both the jelly and vitelline membrane were removed with trypsin. The trypsin treatment consisted of a 30 minute exposure to a 0.05% sea water solution of crude trypsin (M. B. L. stock) pH 8.2 (Tyler and Metz, 1955). Following either treatment, the eggs were washed repeatedly with filtered sea water until the supernatants no longer agglutinated sperm in a 1% suspension.

*Substances released upon fertilization*

Both dejellied and trypsin treated eggs were inseminated with a minimum amount of sperm sufficient to ensure at least 99% fertilization as evidenced by cleavage (final concentration approximately 5 $\mu$l undiluted semen in a 100 ml egg suspension). The concentration of the egg suspensions was about 5–10%, by volume, of freshly shed eggs. Equal volumes of eggs in all experiments were set aside as uninseminated controls. Ten minutes after insemination the supernatant sea water was decanted from the fertilized and unfertilized eggs. Sperm were removed from the former supernatant by centrifugation at 4° C for 10 minutes at 12,000 × g. Prior to further treatment, supernatants collected from both unfertilized and fertilized eggs were tested for sperm agglutinating activity. If the supernatants of the unfertilized eggs agglutinated sperm, the experiment was terminated and the solutions discarded.

*Substances released upon fertilization membrane formation*

In experiments involving artificially induced formation of the fertilization membrane only dejellied eggs were used. After removal of the jelly and thorough washing in sea water, these eggs were allowed to settle through millipore-filtered (0.45 $\mu$ pores) sea water into a more dense layer of an isosmotic sucrose-glycerol solution. This solution consisted of one volume of 1.08 *M* sucrose and four volumes of 1.08 *M* glycerol (Faust, Jones, and Parpart, 1959). After removal of the eggs by light centrifugation, the sucrose-glycerol solution was dialyzed against sea water (4° C) and then tested for sperm agglutinating activity. Eggs treated in this way were observed in the light microscope after removal from the sucrose-glycerol solution. They all exhibited what appeared to be a fertilization membrane. Although systematic counts were not made, a few eggs went through at least one cleavage.

*Preparation of the egg jelly solutions*

Solutions of egg jelly, obtained by washing unfertilized eggs in acid sea water were concentrated by precipitation from sea water with 2 volumes of cold ethanol. After at least 12 hours in the cold, the precipitate was redissolved and dialyzed

against distilled water. The egg jelly used in the Ouchterlony plates contained approximately 50 μg fucose/ml according to the primary cysteine reaction (PCyR1) of Dische, Shettles and Osnos (1949).

*Concentration of substances released from eggs*

Following sperm agglutination tests, all of the supernatant sea water from the fertilized, unfertilized, and artificially activated egg suspensions was dialyzed against running tap water for 12 hours followed by distilled water for 24 hours, with frequent changes, at 4° C. These solutions were concentrated approximately thirty-fold in a Rinco rotary evaporator at about 30° C. Concentration of all solutions by pervaporation or lyophilyzation was equally effective. After concentration, the preparations were stored at −20° C.

*Preparation of antibodies*

Antibodies were prepared in 2 rabbits against the nondialyzable components present in the concentrated supernatants of dejellied, fertilized eggs. One and one-half milliters of a distilled water solution of the material (43 μg/ml of Nessler nitrogen) were emulsified with an equal volume of Freund's complete adjuvant (Difco) and administered as a single subcutaneous injection. Heart bleedings were made during the eighth week after injection. The antigenic components which were released from the eggs at fertilization were designated cortical response antigens (CRA). In addition to the anti-CRA, antibodies against other egg components were prepared in rabbits employing Freund's complete adjuvant, subscapular injections, and ear bleedings. These antibodies were prepared against dejellied unfertilized eggs (6608) and egg jelly (6612, 4/7/67; A-12, 2/20/69; A-13, 2/20/69).

*Qualitative identification of antigens*

The Ouchterlony method of double diffusion in agar was used for qualitative identification of antigens. The agar gel consisted of 1% agarose (Seakem, Bausch and Lomb Co.) dissolved in 0.85% sodium chloride containing 0.2% $NaN_3$ and 0.05% $CdCl_2$ (see Crowle, 1961). Six milliliters of melted agarose solution were pipetted onto Kodak slide cover glasses (2″ × 2″). The wells were 7 mm in diameter with a volume of about 0.1 ml and were filled only once. These Ouchterlony plates were incubated in moist chambers at room temperature for about a week. Periodic drawings were made during the incubations. When the precipitin patterns showed no further changes the plates were rinsed in running tap water for at least 24 hours prior to drying at room temperature. When dry, the plates were stained with azocarmine G (Crowle, 1961) and used as transparencies in preparing photographic records and final drawings of the precipitin patterns.

## Results

*Release of sperm agglutinin*

When a drop of a dilute sperm suspension was mixed with a drop of sea water in which dejellied or trypsin treated eggs had been fertilized the sperm immediately

TABLE I

*Failure of sperm to reagglutinate after pretreatment with either fertilizin or cytofertilizin.* Plus (+) and minus (−) refer, respectively, to the presence or absence of agglutination*

| Solution mixed with sperm | Sperm pretreatment | | |
|---|---|---|---|
| | 0.05 ml of 5% sperm suspension plus 0.15 ml SW (no agglutination) | 0.05 ml of 5% sperm suspension plus 0.15 ml CRA titer = 9 (agglutinated and reversed) | 0.05 ml of 5% sperm suspension plus 0.15 ml EJ titer = 27 (agglutinated and reversed) |
| 0.05 ml EJ (titer = 27) | + | − | − |
| 0.05 ml CRA (titer = 9) | + | − | − |
| Sea water | − | − | − |

* The egg jelly (EJ) preparation used in this experiment was prepared by diluting a pH 5.0 sea water wash of eggs. The original sperm agglutinating titer of the EJ solution obtained in this way was about 20,000. The sperm agglutinating titer of the cortical response antigen (CRA) preparation obtained upon fertilizing half of these eggs was about 9. The pH of both EJ and CRA preparations when mixed with sperm was the same pH as that of the SW used.

agglutinated. This agglutination was weak and reversed rapidly. A weak, reversible sperm agglutination was also observed when a drop of a sea water solution of the material released by the artificially activated eggs was mixed with a drop of a sperm suspension. The supernatant sea water of unfertilized dejellied control eggs did not induce sperm agglutination.

*Agglutination inhibition experiments*

In an attempt to distinguish between fertilizin induced sperm agglutination and the agglutination induced by the supernatants of fertilized eggs, agglutination inhibition experiments were performed. Sperm suspensions were agglutinated by approximately equal concentrations of one or the other agglutinin, and the agglutination was allowed to reverse. Sperm suspensions treated in this way did not reagglutinate upon the further addition of either agglutinin. This experiment was repeated twice using different preparations with at least 5 replicate runs each time. Consistent results were obtained and the results of one such experiment are detailed in Table I.

*Qualitative identification of the antigens*

Whole serum from rabbits immunized against egg jelly (6612, A-12, A-13), regularly formed 4 precipitin bands when diffused in agar against concentrated egg jelly solutions. All 4 bands were continuous with 4 bands formed between antijelly and the CRA preparations from both dejellied and trypsin treated eggs as shown in Figure 1.

Of these 4 precipitin bands, 3 were observed between antijelly (6612) and the concentrated supernatants of unfertilized acid dejellied as well as trypsin treated eggs (Figure 2). In one of the concentrated supernatants of trypsin treated unfertilized eggs there was a trace of a fourth precipitin band.

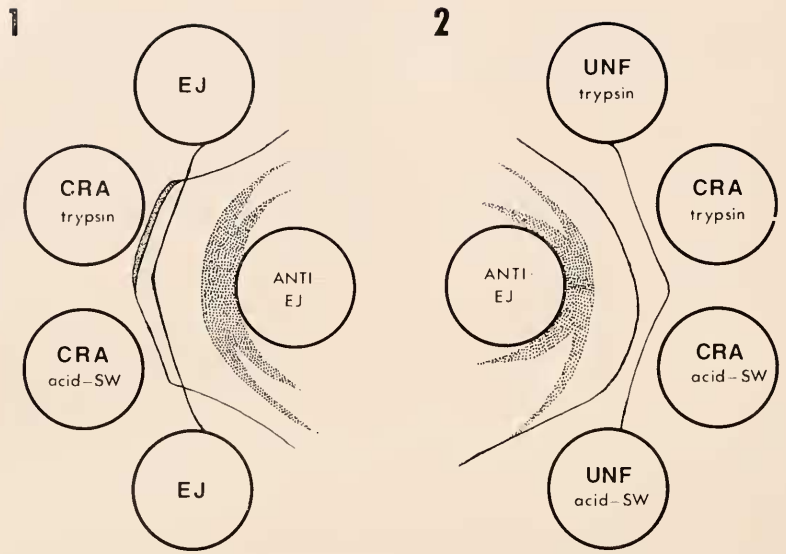


FIGURE 1. Ouchterlony agar diffusion test. The antibody well contained 0.1 ml anti-egg jelly antiserum (6612). The other wells were filled with 0.1 ml of various antigen preparations. EJ: egg jelly obtained by washing eggs with acidified sea water concentrated by alcohol precipitation. CRA trypsin: cortical response antigen preparation (concentrated 100-fold) from trypsin treated eggs. CRA acid-SW: cortical response antigen preparation (concentrated 100-fold) from eggs treated with acid sea water for removal of jelly.

FIGURE 2. Ouchterlony agar diffusion test. The antibody well contained 0.1 ml anti-egg jelly antiserum (6612). The other wells were filled with 0.1 ml of various antigen preparations. CRA trypsin: cortical response antigen preparation (concentrated 100-fold) from trypsin treated eggs. CRA acid-SW: cortical response antigen preparation (concentrated 100-fold) from eggs treated with acid sea water for removal of jelly. UNF trypsin: supernatant of unfertilized trypsin treated eggs (concentrated 100-fold). UNF acid-SW: supernatant of unfertilized eggs (concentrated 100-fold) which had been dejellied with acidified sea water.

When anti CRA (A-5) was diffused against a sample of the CRA preparation (from acid SW-dejellied eggs) which was originally injected into the rabbits, as many as 11 precipitin bands have been observed (Figs. 3 and 4). At least 10 of the 11 precipitin bands were continuous with 10 bands formed between anti-CRA (A-5) and the CRA preparations from fertilized trypsin treated eggs, as well as artificially activated eggs (Fig. 4). When the CRA preparations from acid sea water and trypsin treated eggs were diffused toward antiserum prepared against dejellied egg homogenates (6608) all the precipitin bands of the 2 antigen preparations were continuous (Fig. 4).

All of the conclusions drawn from observations of double diffusion plates were based on precipitin patterns which were duplicated at least once. The same antigen preparations from eggs of different animals yielded identical precipitin patterns. Some antigen preparations from single animals were concentrated in different ways and found to have identical precipitin patterns. However, the possibility that some precipitin bands represent artifacts introduced during concentration or immunodiffusion has not been rigorously excluded.

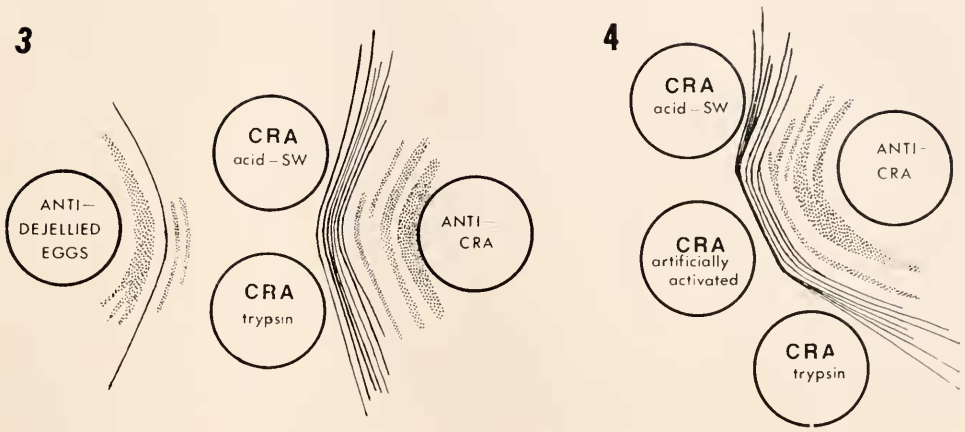


FIGURE 3. Ouchterlony agar diffusion test. Anti-dejellied eggs: antiserum (6608) prepared against homogenates of eggs which had been dejellied with acidified sea water. Anti-CRA: antiserum (A-5) prepared against a cortical response antigen preparation from eggs which had been dejellied with acidified sea water. CRA acid-SW: cortical response antigen preparation from eggs which were dejellied with acidified sea water (concentrated 60-fold). CRA trypsin: cortical response antigen preparation from trypsin treated eggs (concentrated 30-fold).

FIGURE 4. Ouchterlony agar diffusion test. Anti-CRA: antiserum (A-5) prepared against cortical response antigens from eggs which had been dejellied with acidified sea water. CRA-acid SW: cortical response antigen preparation from eggs which had been dejellied with acidified sea water (concentrated 60-fold). CRA artifically activated: cortical response antigen preparation from eggs which had been treated with a sucrose-glycerol solution as a means of artificially inducing fertilization membrane formation (approximately 30-fold concentration). CRA trypsin: cortical response antigen preparation from trypsin treated eggs (concentrated 30-fold).

## DISCUSSION

### *Identification of cytofertilizin*

Motomura (1950) introduced the term "cytofertilizin" in referring to a sperm agglutinin released from eggs of several species of Japanese sea urchins. In repeating some of Motomura's experiments, Byers (1951) failed to detect the release of a sperm agglutinin from dejellied eggs of *Arbacia punctulata*. However, the results of the present work on the same species clearly demonstrate a sperm agglutinin in the supernatant sea water of dejellied eggs following the cortical response at fertilization or artificial activation.

### *Similarity of cytofertilizin and fertilizin*

Since both cytofertilizin and fertilizin agglutinate sperm of the species and since both reactions reverse spontaneously, the mechanisms of agglutination and the agglutinins themselves appear to be similar. This similarity of the agglutinins was confirmed in the agglutination inhibition experiments (Table I). The latter

are interpreted to indicate that cytofertilizin and fertilizin combined with the same site on the sperm surface. The reversal of agglutination has been explained as a breakdown of the multivalent agglutinin into univalent fragments (Tyler, 1941; Stern, 1967).

Additional evidence for similarity of the agglutinins was obtained using immunological techniques. Since anti-jelly antiserum precipitated the agglutinin from egg jelly preparations as well as CRA preparations, it was concluded that both agglutinins were antigenic and similar. The immunodiffusion studies indicated that fertilizin and cytofertilizin were antigenically identical since all 4 egg jelly antigens were present in CRA preparations. The supernatants of unfertilized dejellied as well as trypsin treated eggs lacked one of these 4 antigens. The absence of sperm agglutinating activity in these supernatants, correlated with the absence of one antigen, suggests that this antigen is the sperm agglutinin. In the one case in which the supernatant of unfertilized trypsin treated eggs contained a trace of this fourth antigen, some cytolysis could have occurred. The trypsin treated eggs were much more fragile than acid dejellied eggs and tended to cytolyze with moderate mechanical agitation.

It has been previously reported that cytofertilizin and fertilizin were chemically and functionally distinct. Motomura (1950) inferred that the solubilities of the two substances, as a function of pH, were sufficiently different that they should be considered separate chemical entities. Hagström (1956), using other species, was not able to confirm these pH-dependent solubility relationships but he did conclude that cytofertilizin was different from fertilizin because the two had different effects on the fertilization rate.' It should be pointed out that both workers did not attempt to determine the purity of their preparations. Consequently the differing properties of their preparations of cytofertilizin and fertilizin could easily have been due to the presence of contaminating substances in either preparation. The present work indicates that there is as yet no good evidence to support the claim that cytofertilizin and fertilizin are different substances.

It is of interest that concentrated egg jelly solutions contained at least 4 antigenic macromolecules, none of which was unique to the egg jelly preparations. These results contrast with those from other species that indicate an antigenic component(s) in the egg jelly which is not found in the egg (Perlmann and Perlmann, 1957; also, see discussion, Metz, 1968).

*Diffusion of an antigen from the egg surface*

Assuming that the sperm agglutinin represents one antigen, the different CRA preparations contained at least 9 other antigens which precipitate with anti-CRA serum. At least 3 of these antigens were released by unfertilized, dejellied eggs. These antigens apparently diffused from the egg surface and did not require the cortical response to fertilization for release. Accordingly, these antigens were not considered to be cortical response antigens or permanent components of egg jelly. A non-permanent antigenic component of egg jelly has previously been described by Baxandall, Perlmann, and Afzelius (1964) and by Perlmann and Perlmann (1957). These antigens may also be similar to the non-agglutinating component of egg jelly to which Messina and Monroy (1956) referred.

*Relationship between CRA and the fertilization product*

Ishihara (1964, 1968ab) investigated the chemical nature of the organic substances in the supernatant sea water of inseminated eggs. He designated these substances the "fertilization product," assuming that they were released from the egg upon fertilization, concomitant with the appearance of the fertilization acid. Since Ishihara's chemical analyses were performed on hydrolysates of the "fertilization product," his data could not distinguish among its various macromolecular components. The results of the present work have shown that the preparation corresponding to the "fertilization product" in this species of urchin consists of at least 3 antigens released before fertilization and 7 or more antigens (CRA) released following fertilization or artificial activation. While no direct evidence has been presented, it would seem likely that some or all of the cortical response antigens are released from the cortical granules when they rupture during the cortical response.

The help of Dr. A. A. Humphries in the preparation of some of the antibodies used in this study is gratefully acknowledged.

## Summary

1. The supernatant sea water of inseminated dejellied or trypsin treated *Arbacia punctulata* eggs was shown to contain a sperm agglutinin which was released from the eggs after insemination. This sperm agglutinin has previously been called "cytofertilizin" in other species.

2. A sperm agglutinin was also released from dejellied eggs in which the formation of a fertilization membrane had been artificially induced.

3. Agglutination inhibition experiments suggested that the agglutinin released from dejellied fertilized eggs (cytofertilizin) and the agglutinin from egg jelly (fertilizin) bind to the same sperm receptor sites.

4. Egg jelly preparations were found to contain at least 4 antigenically distinct macromolecules. At least 1 of the egg jelly antigens was the sperm agglutinin, fertilizin.

5. On the basis of double diffusion studies using antiserum against egg jelly, the 4 antigens in egg jelly preparations were indistinguishable from 4 antigens released from dejellied eggs during the cortical response to fertilization (cortical response antigens).

6. At least 3 antigens were released from dejellied or trypsin treated unfertilized eggs. These antigens were also present in egg jelly preparations and cortical response antigen preparations, but did not induce sperm agglutination.

7. Double diffusion studies using antiserum against cortical response antigens indicated that at least 7 antigens were released from dejellied eggs when they were inseminated or when the formation of the fertilization membrane was artificially induced. The same antigens were released from trypsin treated eggs after insemination.

8. The significance of these findings is discussed in relation to cytofertilizin and the fertilization product. The results indicate that cytofertilizin and fertilizin are functionally and antigenically identical.

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